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# Covalent bonding of heparin to a vinyl copolymer for biomedical applications

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In order to prepare polymer surfaces of vinyl type, provided with long-term haemocompatibility, a commercial ethylene–vinyl alcohol copolymer (EVAL) was covalently heparinized, employing two different bifunctional reagents (adipoil chloride and hexamethylene diisocyanate). The amount and activity of the heparin bonded to the polymer films were evaluated as a function of the concentration of the heparin solutions employed. Also, the influence exerted by the presence of various hydrophilic 'spacing arms' of different molecular weights, either neutral or provided with electrical charge, was investigated. By *in vitro* measurements of activated partial thromboplastin time it was seen that all the heparinized samples possessed a high degree of haemocompatibility. For the sake of comparison, heparin was also bonded ionically to EVAL functionalized by introduction of quaternary ammonium groups bonded covalently (by adipoil chloride) to the hydroxyl groups of the polymer. It was seen that the covalent immobilization system provides the polymer surfaces with a superior haemocompatibility. 

*Keywords: Haemocompatibility, heparin, covalent binding, ethylene–vinyl alcohol copolymers*

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A major problem connected with the employment of extra-corporeal apparatus, like those used in haemodialysis, is the formation of thrombi during the clinical procedure. In order to avoid this, an anticoagulant (usually heparin) is administered to patients before treatment. Hollow fibres made of ethylene–vinyl alcohol copolymers (EVAL) are at present largely employed for the manufacture of haemodialysis filters[1]. They are provided with excellent mechanical properties and fairly good hydrophilicity, due to the high percentage content of vinyl alcohol in the copolymer. Since their haemocompatibility is less than optimal, some research aimed at its improvement was carried out. For this purpose, heparin was ionically bonded to positively charged groups of different types of polymers[2–6]. In the case of EVAL, this was made by reacting its surface with an $\omega$-dialkyl amino-aldehyde, forming acetalic bonds with the hydroxyl groups of the copolymer[7]. It was seen, however, that the better haemocompatibility of the treated EVAL fibres was due to the progressive release of heparin into the blood, due to the poor stability of its ionic bonds with the polymer surface.

Since heparin proved to be even more effective when covalently bonded to polymer surfaces[8–12], in order to obtain EVAL surfaces able to maintain their haemocompatibility for a long time (and so suitable for employment in permanent or semipermanent prostheses), we have covalently bonded heparin to the functional groups present in EVAL. This reaction was carried out employing two different bifunctional reagents, and also two different 'spacing arms' (able to provide heparin with higher conformational freedom and a more hydrophilic environment) were used.

The influence of the heparin concentration in the reacting solution on the amount and biological (*in vitro*) activity of the bonded heparin was also evaluated. Moreover, the biological activity of covalently bonded heparin was compared to that of heparin ionically bonded to the same matrix. For this purpose, heparin was bonded to EVAL films modified through the insertion of quaternary ammonium groups, able to form electrostatic bonds with the strongly anionic groups of heparin.

## MATERIALS AND METHODS

The commercial EVAL (Kuraray, Japan) polymer employed in this study has a 30:70 molar ethylene–vinyl alcohol ratio. Since an elevated surface–volume ratio is important for the obtainment of reproducible results in *in vitro* tests, the polymer was supported by casting from dimethylformamide (Fluka) onto glass microspheres having an average diameter of 165 $\mu$m. Reactions with the bifunctional binding agents were always carried out under an anhydrous atmosphere, using as solvent formamide (FA; Fluka) refluxed onto $CaSO_4$ (Fluka) and distilled.

Heparin was firstly grafted to EVAL, using adipoil chloride (AC; Fluka) to bind its hydroxyl groups with those of the polymer (*Figure 1A*). The reaction was carried out at 45°C for 3 h under a nitrogen atmosphere, adding to a suspension of polymer-coated glass microspheres (in anhydrous FA) AC and distilled triethylamine (Fluka) in a 1:1 molar ratio, to neutralize

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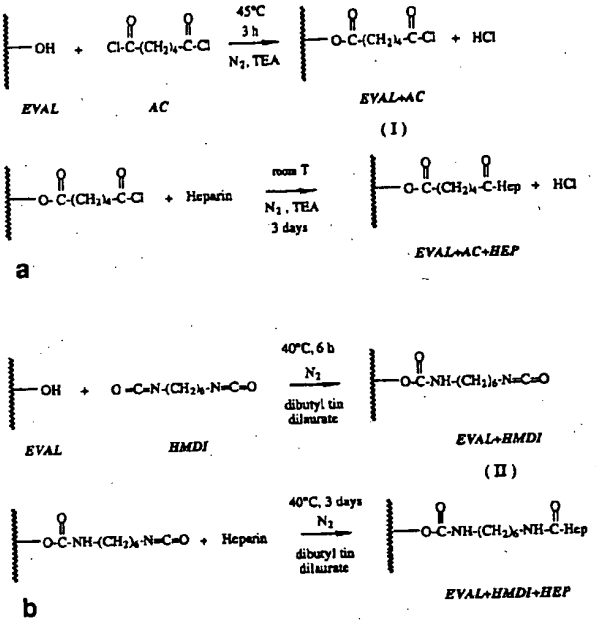


**Figure 1** Reaction scheme 1.

the HCl evolved during the reaction. Heparin (Fluka; 140 IU $mg^{-1}$) was bonded to the functionalized microspheres, reacting FA heparin solutions of different concentration (0.1 and 1%, w/w) for 3 days at room temperature.

In order to evaluate the influence of the type of bond introduced with the coupling agent on the activity of bonded anticoagulant, hexamethylene diisocyanate (HMDI; Fluka), which forms urethane bonds with the hydroxyl groups of heparin, was also used to bind the latter to the EVAL surfaces (*Figure 1B*). The reaction was carried out in the absence of moisture, at 40°C, using dibutyltin dilaurate as catalyst, for about 6 h for the polymer film activation, and then 3 days for the heparin bonding reaction, also employing 0.1 and 1% heparin solutions. After the reactions, the heparinized microspheres were filtered and washed for 24 h at room temperature, firstly with water and then with phosphate-buffered saline (PBS; pH 7.4).

The amount of heparin bonded on the polymer surfaces was determined by difference, utilizing the reaction of heparin with the Toluidine Blue dyestuff (Fluka), whose absorbance was recorded by a double-beam Hitachi U-2000 spectrophotometer operating in the wavelength range 190–1100 nm. The adsorption yield was determined by measuring spectrophotometrically the difference between the amount of heparin in the solution before and after heparinization (adding to this latter term the heparin content of water and PBS washing solutions). The absorbance at 631 nm was recorded. This analytical procedure, described for the evaluation of the heparin content of water solutions, has been modified by us in order to obtain reliable data in the presence of organic solvents[13].

In order to evaluate the influence of both the polymer surface hydrophilicity and the 'spacing' of the heparin molecules on its biological activity, a poly-functional amine (tetraethylene-pentamine, TEPA; Fluka) and a poly-hydroxylated polymer [poly(hydroxyethyl methacrylate), pHEMA, $M_n$ = 2700] were grafted onto the EVAL surface as spacers (*Figure 2*).

TEPA was bonded both via AC and via HMDI, employing 0.1 M TEPA solutions in FA, and the previously described reaction conditions for these reagents, whereas pHEMA was bonded exclusively via HMDI. In both cases the reaction time was 18 h. The heparinization of the functionalized polymer films was made using HMDI, under the above-mentioned experimental conditions.

In order to check that heparin was really chemically bonded and not simply aspecifically adsorbed, after the covalent grafting of heparin the polymer films, both in the presence and absence of the spacing molecule TEPA, washed with water and PBS as reported previously, were evaluated by attenuated total reflection (ATR)/Fourier transform infrared (FT-IR), by employing a Mattson Galaxy 5000 spectrophotometer provided with an ATR device containing either a germanium or zinc selenide crystal.

The *in vitro* biological evaluation of the heparinized microspheres was carried out by contacting them with plasma[13] and measuring the activated partial thromboplastin time (APTT) using an automatic coagulimeter (Koagulab MJ, Ortho Diagnostic System). Heparin was immobilized ionically on EVAL functionalized by reaction with *N,N*-diethylethylenediamine (DED), and using as binding agents both AC and HMDI, with the reaction conditions described previously for TEPA (*Figure 3*). The quaternization reaction of the tertiary ammonium groups of DED to

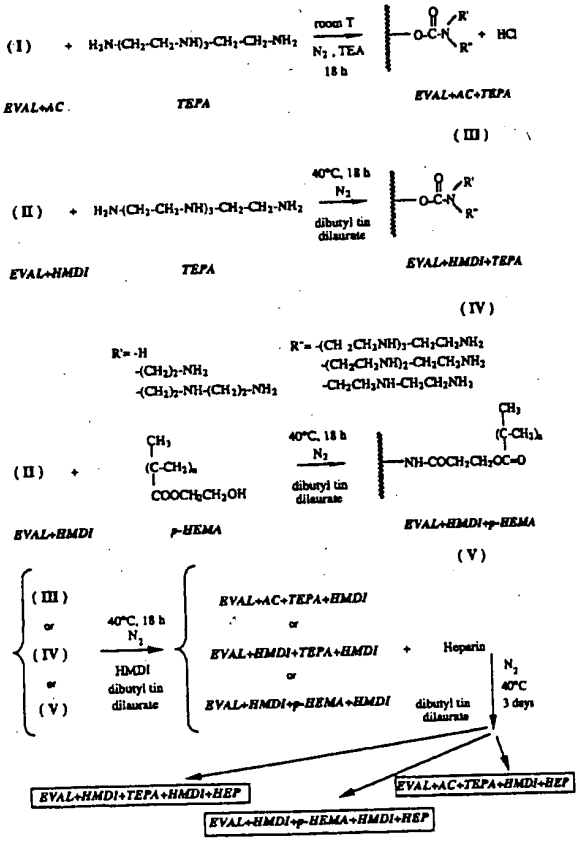


**Figure 2** Reaction scheme 2.

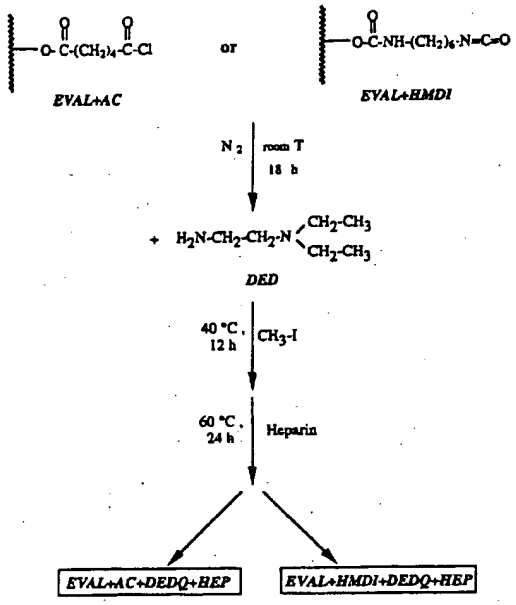


**Figure 3** Reaction scheme 3.

quaternized DED (DEDQ) was carried out in $CHCl_3$ at 40°C for 12 h, with a large excess of $CH_3I$ (Fluka). Under these conditions the reaction yield was quantitative. Some samples were also quaternized using benzyl chloride (Fluka) and then treated with 0.1 M NaOH solution, in order to obtain an exchange reaction with the chloride ions. This solution was added to the washing solution and submitted to determination of chloride ions by ionic chromatography, using an apparatus operating through chemical suppression and conductimetric detection (Dionex 2000i, anionic column AS4A), employing as eluent a mixture of $3.5 \times 10^{-4}$ M $NaHCO_3$ and $3.0 \times 10^{-4}$ M $Na_2CO_3$, with $1.25 \times 10^{-3}$ M $H_2SO_4$ as suppressor. This procedure could not be employed for the determination of iodides, due to insufficient definition of the peaks.

The heparinization (ionic) reaction was realized with aqueous solutions of heparin (0.1 and 1% w/w) at 60°C for 24 h, and after the reaction the microspheres were washed as mentioned above with water and PBS respectively.

## RESULTS AND DISCUSSION

In *Figures 4* and *5* the ATR/FT-IR spectra of the washed polymer films containing heparin bonded directly to EVAL (*Figure 4*) and through the TEPA spacer (*Figure 5*), compared to the corresponding films containing aspecifically adsorbed heparin, are reported. Heparin was detected only when covalently bonded, as shown by the absorptions relevant to the $-SO_3^-$ stretching in the 800–1400 $cm^{-1}$ range, in particular around 1030 and around 1130 $cm^{-1}$. Curve-fitting procedures for the peak centred around 1100 $cm^{-1}$ of *Figure 5* were used to separate bands and to determine their integrated intensities. The fitting was performed with the use of interactive graphic routines. As a result, the area increase of the peak centred at 1040 $cm^{-1}$ in spectrum b can be attributed to the absorption due to the $SO_3^-$ stretching at 1030 $cm^{-1}$. Spectrum a is not

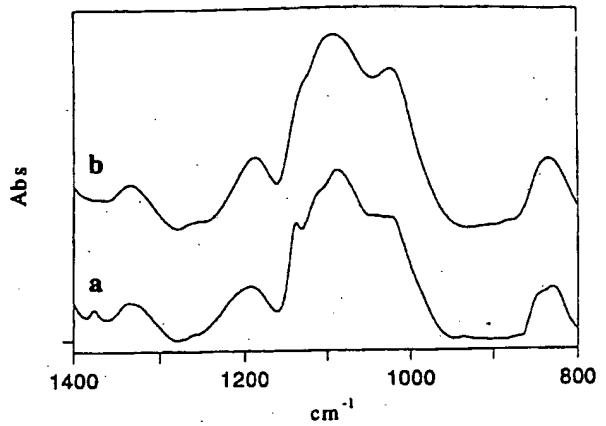


**Figure 4** ATR/FT-IR spectra of washed EVAL–AC films containing aspecific adsorbed heparin (**a**) or covalently bonded heparin (**b**).

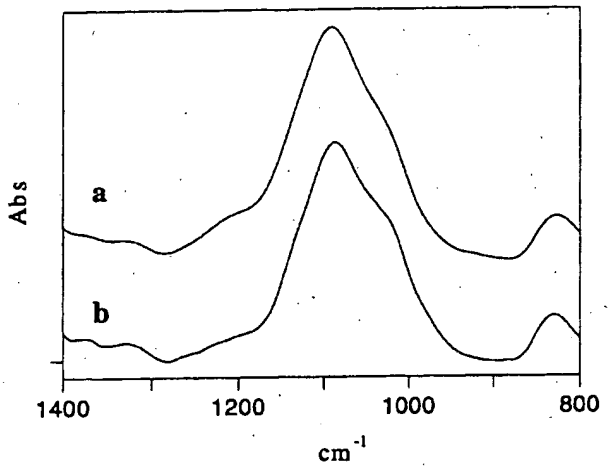


**Figure 5** ATR/FT-IR spectra of washed EVAL–AC–TEPA films containing aspecific adsorbed heparin (**a**) or covalently bonded heparin (**b**).

different from the one obtained for untreated EVAL–AC–TEPA film.

The results relevant to the covalent binding of heparin to EVAL using both AC and HMDI, at two different anticoagulant concentrations, are reported in *Table 1*. It can be observed that the amount of heparin bonded to the films is a function of the concentration of the anticoagulant solution employed, and that the biological activity of the films correlates positively with their heparin content.

As for the influence of the dibutyltin dilaurate

**Table 1** Biological activity of heparin bonded to ethylene–vinyl alcohol copolymer through adipoil chloride or hexamethylene diisocyanate

| Bonding sequence | Bonded heparin ($\mu g\,cm^{-2}$) | APTT (s) |
|---|---|---|
| AC–heparin* | 0.9 ± 0.3 | 266 ± 66 |
| AC–heparin† | 5.4 ± 0.8 | 250 ± 36 |
| HMDI–heparin* | 4.5 ± 1.0 | 63 ± 6 |
| HMDI–heparin† | 50 ± 11 | No clot |

The reference value of APTT for glass microspheres was 30 ± 1 s.
*0.1% heparin solution.
†1% heparin solution.

catalyst on the amount of bonded heparin (when HMDI is used as binding agent), it was seen that when it is absent and 0.1 M heparin solutions are employed, the amount of bonded heparin is much lower (0.7 $\mu g\,cm^{-2}$).

A comparison of the biological activity of microspheres coated with the polymer, heparinized with 1% heparin solution and using the two different bifunctional reagents, the specific heparin content being equal, is given in *Figure 6*. It can be observed that a much higher activity is obtained when AC is used, and this was interpreted as due to the fact that the unreacted AC, when hydrolysed, provides the polymer surface with an acidic character, with consequent electrostatic repulsion among the carboxyl groups and the numerous electronegative groups present in the heparin molecule. This repulsion would project the heparin molecule further off the polymer surface, towards the aqueous phase where the anticoagulant, by interaction with antithrombin III and thrombin, carries out its biological activity.

Moreover, it was seen that in this case the samples containing a lower amount of anticoagulant (i.e. heparinized with a 0.1% solution) proved to be more active (see *Figure 7*), and this unusual behaviour was also attributed to a lower steric hindrance for the interaction of the more 'isolated' heparin molecules with the haematic proteins.

The amount of heparin bonded to the polymer surface when the spacers TEPA and pHEMA were employed, as well as the relevant biological activity, are reported in *Table 2*, while in *Table 3* the biological activities of the films either in the presence or in the absence of the spacers are compared. As a general rule, the spacer enhances the anticoagulant activity, and electrostatic repulsions seem to have the same effect on it.

In *Figure 8* the biological activity of EVAL–HMDI–TEPA films heparinized with solutions containing both 0.1 and 1% (w/w) of heparin is shown. It can be seen that in this case the APTT values are a linear function of the absolute heparin content of the film, to demonstrate that, in this case, the spacer agent (TEPA)

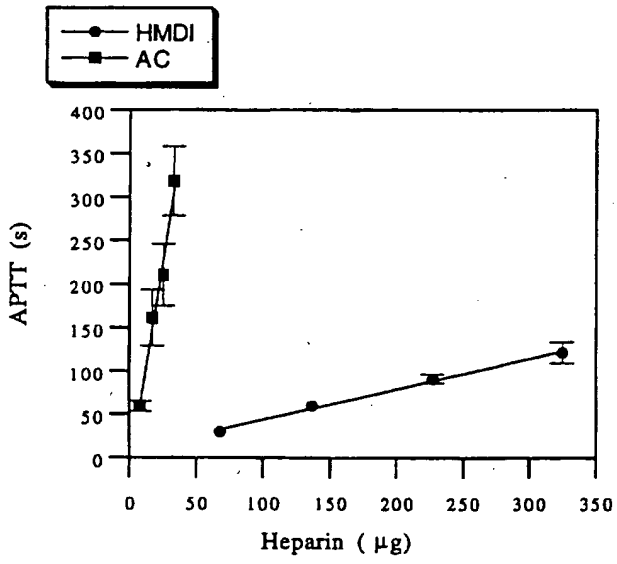


**Figure 6** Activity of heparin bonded onto EVAL by AC or HMDI (1% heparin solutions were employed).

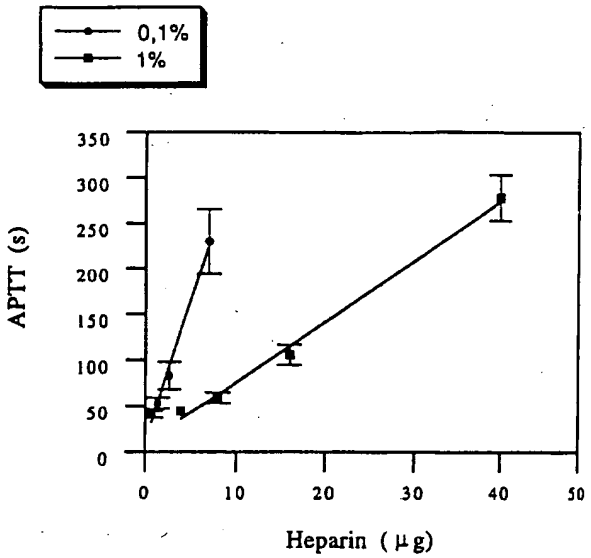


**Figure 7** Biological activity of heparin bonded onto EVAL by AC using 0.1 or 1% (w/w) heparin solutions.

**Table 2** Biological activity of heparin bonded to ethylene–vinyl alcohol copolymer surfaces by adipoil chloride or hexamethylene diisocyanate and using tetraethylene-pentamine as spacer

| Bonding sequence | Bonded heparin ($\mu g\,cm^{-2}$) | APTT (s) |
|---|---|---|
| AC–TEPA–HMDI–heparin* | 1.6 ± 0.7 | 96 ± 17 |
| AC–TEPA–HMDI–heparin† | 27 ± 7 | No clot |
| HMDI–TEPA–HMDI–heparin* | 1.9 ± 0.4 | 230 ± 100 |
| HMDI–TEPA–HMDI–heparin† | 23 ± 8 | No clot |
| HMDI–pHEMA–HMDI–heparin† | 50 ± 1 | No clot |

*0.1% heparin solution.
†1% heparin solution.

**Table 3** Influence of spacer type on the biological activity of heparin (1% solution) bonded to ethylene–vinyl alcohol copolymer

| Bonding sequence | Total amount of heparin in the sample (μg) | APTT (s) |
|---|---|---|
| HMDI–pHEMA–HMDI–heparin | 38 ± 0.8 | 63 ± 4 |
| AC–TEPA–HMDI–heparin | 40 ± 10 | 85 ± 8 |
| HMDI–TEPA–HMDI–heparin | 35 ± 10 | 380 ± 40 |
| HMDI–heparin | 40 ± 11 | 28 ± 2 |
| AC–heparin | 40 ± 5 | 275 ± 25 |

is able to neutralize completely (differently from the previously examined situations) any steric hindrance effect. pHEMA proved to be a less efficient spacer agent than TEPA (*Table 3*), probably due to the formation of a 'multibond system' between the numerous functional groups of heparin and spacer, bringing about severe limitations in heparin's conformational freedom.

When we compare the biological activity of polymer films heparinized (through AC) without spacer with that of films heparinized using TEPA as spacer (*Figure 9*), the results show clearly that, in spite of the spacer agent, the activity is lower for the latter ones. We interpreted this as a neutralization (by the TEPA basic

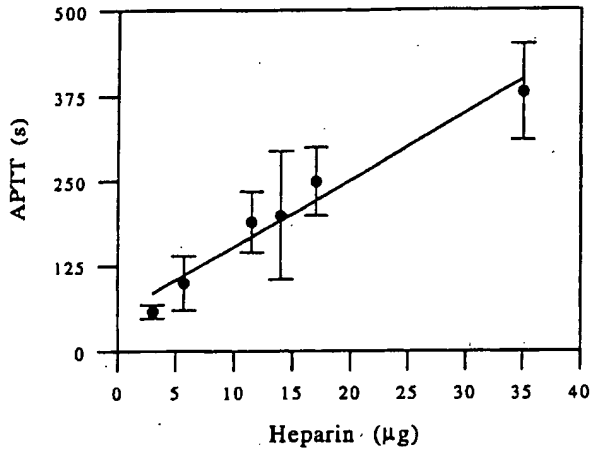


**Figure 8** Biological activity of heparin bonded onto EVAL–HMDI–TEPA by HMDI.

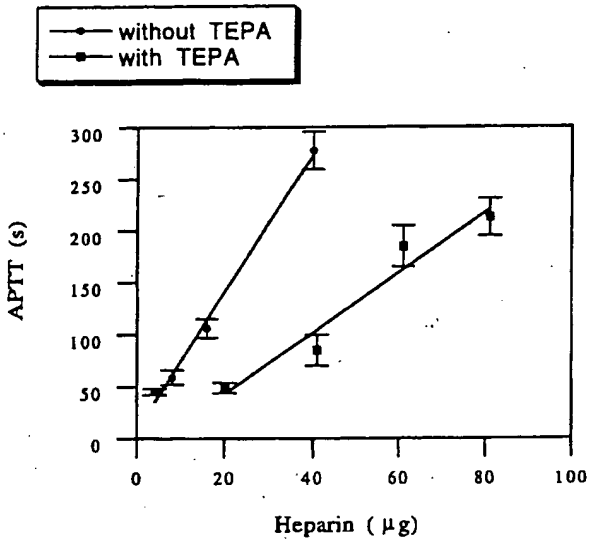


**Figure 9** Influence of the spacer TEPA on the activity of heparin bonded covalently to EVAL by AC (1% heparin solutions) either without (●) or with (■) spacer.

groups) of the surface acidity, brought about by the adipic acid formed by AC hydrolysis, with consequent loss of the above-mentioned electrostatic repulsion effect induced by the acidic polymer surface on the grafted heparin molecules.

Furthermore, when the heparinization was carried out with 1% (w/w) heparin solutions, and using for the APTT tests an amount of polymer-coated microspheres such that the total heparin qualities were comparable, it was observed that the biological activity was much higher when the grafting of TEPA onto EVAL was made through HMDI rather than through AC (see *Table 3*, lines 2 and 3 respectively).

Since we have ascertained that the amount of bonded TEPA is much higher with HMDI than with AC, we inferred that in the former case the successively grafted heparin would have a higher probability of bonding to a TEPA residue that has not already reacted with another heparin molecule; this fact would provide the anticoagulant molecule with a higher steric freedom.

In *Table 4* we report the data obtained for films containing heparin ionically bonded to DEDQ at two different concentrations; the grafting of DEDQ to the EVAL-coated microspheres was also effected through both AC and HMDI.

In *Figure 10*, a comparison between covalently and ionically bonded heparin is given. The higher activity of the latter was considered to be due to heparin release during the duration time (about 10 min) of the APTT test; this would be demonstrated by the fact that the difference of activity becomes more remarkable for higher contents of bonded (both ionically and covalently) anticoagulant, where the release phenomena can play a more important role. However, in a comparison between samples heparinized either ionically or covalently (with TEPA as spacer and HMDI as binding agent), the latter proved to be much more active (see *Figure 11*).

## CONCLUSIONS

In the present study a positive correlation between the biological activity of heparinized films (evaluated by APTT measurements) and their heparin content was observed. Moreover, their heparin content is a function of the concentration of the solution employed for the heparinization.

**Table 4** Heparin bonded ionically to ethylene–vinyl alcohol copolymer by either adipoil chloride or hexamethylene diisocyanate

| Bonding sequence | Bonded heparin ($\mu g\,cm^{-2}$) | APTT (s) |
|---|---|---|
| AC–DEDQ–heparin* | 0.7 ± 0.2 | 70 ± 19 |
| AC–DEDQ–heparin† | 11 ± 2 | No clot |
| HMDI–DEDQ–heparin* | 1.0 ± 0.2 | 70 ± 19 |

*0.1% heparin solution.
†1% heparin solution.

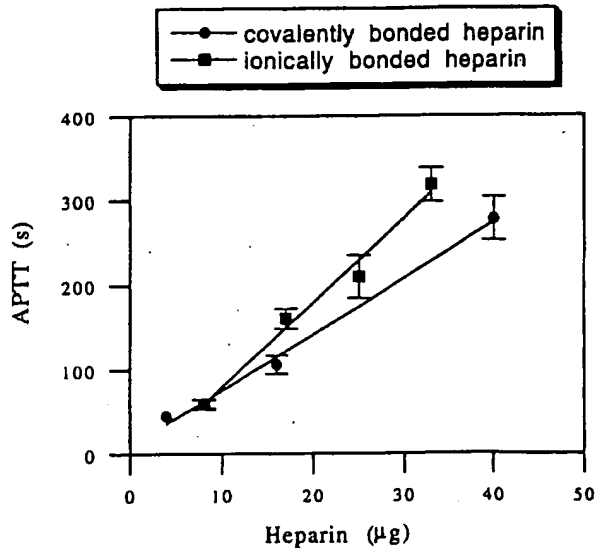


**Figure 10** Activity of heparin (1% solutions) bonded to EVAL by AC either covalently (●) or ionically (■).

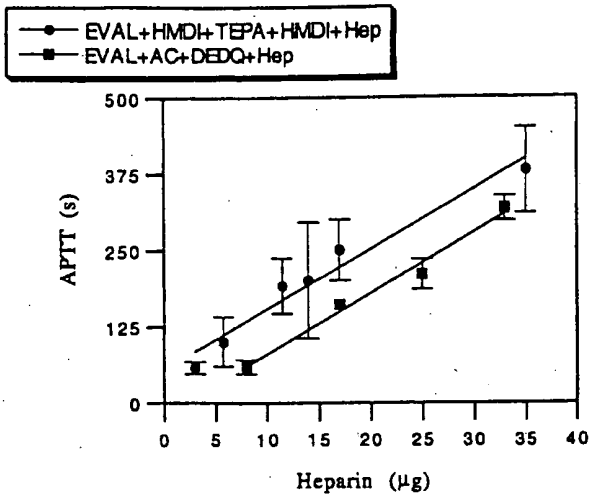


**Figure 11** Biological activity of heparin bonded ionically (by AC) and covalently with TEPA spacer and 1% heparin solutions.

When heparin was bonded directly to EVAL films through AC, its activity was higher than when HMDI was employed, presumably because of repulsive interactions among heparin molecules and the surface acidic groups. In the absence of these electrostatic repulsions, the presence of a spacer agent increases the biological activity of the bonded heparin. It was seen that, when pHEMA was used as spacer, its effect was lower than that exerted by TEPA, and this was attributed to the formation of covalent 'multibonds' with heparin, with consequent limitation of its conformational freedom.

A higher activity was remarked for ionically heparinized films with respect to covalent bonding, and this was attributed to a partial heparin release into the test solution. However, when TEPA was used as spacer and HMDI as the bonding agent, covalently heparinized EVAL films showed a remarkably higher activity, the total heparin content of the samples being equal.

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